TITLE PAGE:
Authors:

Roque Serrano (ORCID id: 0000-0002-0700-8369)
Research Institute for Pesticides and Water (IUPA), University Jaume I, Av. Sos Baynat S/N, 12071, Castellón, Spain. Research Unit of Marine Ecotoxicology (IATS-IUPA), Ribera de Cabanes, S/N, 12595, Cabanes, Castellón, Spain.
Juan Carlos Navarro: (ORCID id: 0000-0001-6976-6686)
Institute of Aquaculture Torre de la Sal (IATS-CSIC), Ribera de Cabanes, S/N, 12595, Cabanes, Castellón, Spain. Research Unit of Marine Ecotoxicology (IATS-IUPA), Ribera de Cabanes, S/N, 12595, Cabanes, Castellón, Spain.
Tania Portolés (ORCID id: 0000-0002-8422-0822)
Research Institute for Pesticides and Water (IUPA), University Jaume I, Av. Sos Baynat S/N, 12071, Castellón, Spain.
Carlos Sales (ORCID id: 0000-0001-6337-063X)
Research Institute for Pesticides and Water (IUPA), University Jaume I, Av. Sos Baynat S/N, 12071, Castellón, Spain.
Joaquin Beltran (ORCID id: 0000-0003-4387-101X)
Research Institute for Pesticides and Water (IUPA), University Jaume I, Av. Sos Baynat S/N, 12071, Castellón, Spain.
Oscar Monroig (ORCID id: 0000-00001-8712-0440)
Institute of Aquaculture Torre de la Sal (IATS-CSIC), Ribera de Cabanes, S/N, 12595, Cabanes, Castellón, Spain. Research Unit of Marine Ecotoxicology (IATS-IUPA), Ribera de Cabanes, S/N, 12595, Cabanes, Castellón, Spain.
Félix Hernández (ORCID id: 0000-0003-1268-3083)
Research Institute for Pesticides and Water (IUPA), University Jaume I, Av. Sos Baynat S/N, 12071, Castellón, Spain. Research Unit of Marine Ecotoxicology (IATS-IUPA), Ribera de Cabanes, S/N, 12595, Cabanes, Castellón, Spain.

TITLE: Identification of new, very long-chain polyunsaturated fatty acids in fish by gas chromatography coupled to quadrupole/time-of-flight mass spectrometry with atmospheric pressure chemical ionization

Keywords: gas chromatography, quadrupole/time-of-flight mass spectrometer, very long-chain polyunsaturated fatty acids, fish.

Corresponding author information:
Roque Serrano
Research Institute for Pesticides and Water (IUPA), University Jaume I, Av. Sos Baynat S/N, 12071, Castellón, Spain. **e-mail**. serrano@uji.es. **phone**: +34 964387358

Declarations

Funding

Pla 2018 de Promoció de la Investigació a l'UJI. Research Project: Identificación y cuantificación de ácidos grasos poliinsaturados de cadena muy larga mediante cromatografía de gases acoplada a espectrometría de masas de alta resolución en peces. Ref: UJI-B2018-08. Programa Estatal de Investigación, Desarrollo e Innovación orientada a los retos de la sociedad (Ministry of Economy and Competitiveness): Research Project "Ácidos grasos poliinsaturados de cadena muy larga en peces: biosíntesis e implicaciones durante fases tempranas de desarrollo en especies cultivadas" (AGL2013-40986-R). Ramon y Cajal Program from the Ministry of Economy and Competitiveness, Spain (RYC-2017-22525). The UJI authors acknowledge the financial support from Generalitat Valenciana (Group of Excellence Prometeo 2019/040).

Conflict of interest

There are no conflicts to declare.

Availability of data and material

Data and material are available

Code availability

Not applicable

Identification of new, very long-chain polyunsaturated fatty acids in fish by gas chromatography coupled to quadrupole/time-of-flight mass spectrometry with atmospheric pressure chemical ionization

Roque Serrano*[a,b], Juan Carlos Navarro[b,c], Tania Portolés[a], Carlos Sales[a], Joaquín Beltrán[a], Óscar Monroig[b,c], Félix Hernández[a,b]

[(a)] *Research Institute for Pesticides and Water (IUPA), University Jaume I, Av. Sos Baynat S/N, 12071, Castellón, Spain.*
[(b)] *Research Unit of Marine Ecotoxicology (IATS-IUPA), Ribera de Cabanes, S/N, 12595, Cabanes, Castellón, Spain.*
[(c)] *Instituto de Acuicultura de Torre de la Sal (IATS-CSIC), Ribera de Cabanes, S/N, 12595, Cabanes, Castellón, Spain.*

Abstract

The characterization of very long-chain (>C24) polyunsaturated fatty acids (VLC-PUFAs), which are essential in the vision, neural function and reproduction of vertebrates, is challenging because of the lack of reference standards and their very low concentrations in certain lipid classes. In this research, we have developed a new methodology for VLC-PUFA identification based on gas chromatography coupled to quadrupole/time-of-flight mass spectrometry with an atmospheric pressure chemical ionization source (GC-APCI-QTOF MS). The mass accuracy attainable with the innovative QTOF instrument, together with the soft ionization of the APCI source, provides valuable information on the intact molecule, traditionally lost with electron ionization sources due to the extensive fragmentation suffered. We have identified, for the first time, VLC-PUFAs with chains up to 44 carbons in eyes, brain and gonads of gilthead sea bream, a commercially important fish in the Mediterranean. The added value of ion mobility-mass spectrometry (IMS), recently developed in combination with GC-QTOF MS, and the contribution of the collisional cross-section (CCS) parameter in the characterization of novel VLC-PUFAs (for which reference standards are not available) have been also evaluated. The methodology developed has allowed assessing qualitative differences between farmed and wild fish, and opens new perspectives in a still scarcely known field of research.

Keywords: gas chromatography, quadrupole/time-of-flight mass spectrometer, very long-chain polyunsaturated fatty acids, fish.

Corresponding author information: Roque Serrano. e-mail. serrano@uji.es, phone: +34 964387358

Introduction

The existence of very long-chain polyunsaturated fatty acids (VLC-PUFAs) ranging from 26 to 36 carbons in length in mammal spermatozoa and vertebrate retina was reported in 1986-87 by Poulos et al. [1,2] and Aveldaño and Sprecher [3]. Since then, low concentrations of up to C38 VLC-PUFAs have been reported in phosphatidylcholine (PC) of human brain [4]; triacylglycerides (TG) and cholesterol esters (CHE) of mammalian testis [5]; sperm and brain of mammals; PC, sphingomyelin (SM) and cerebrosides (CE) of human retina [6-9], and PC of fish retina [10,11]. VLC-PUFAs have been associated with macular dystrophy [6] and Zellweger's syndrome [4], and are considered essential for the development of the nervous system, retinal function and reproduction in vertebrates [10,12-14]. It is widely accepted that, rather than deriving from diet, VLC-PUFAs are produced endogenously (biosynthesis) by successive elongations from C20 and C22 PUFAs, where eicosapentaenoic acid (C20:5n-3) arises as a preferred substrate for elongation to VLC-PUFAs over C20:4n6 (arachidonic acid) and C22:6n3 (docosahexaenoic acid) [7,15]. The elongation of very long-chain fatty acid protein 4 (Elovl4) plays a major role in VLC-PUFA biosynthesis in vertebrates [6,7,10,16]. Notably, mutations in the murine Elovl4 gene reduce the content of VLC-PUFAs in the retina, leading to vision loss [17]. Fish Elovl4 also participate in VLC-PUFA metabolism and are able to elongate C20 and C22 PUFAs to polyenes of up to 36 carbons [16]. Moreover, the pattern of *elovl4* expression shows that VLC-PUFA metabolism in fish occurs primarily in the brain, eye and gonads [18-21], where these compounds may exert pivotal biological functions despite their relative low abundance in comparison to their shorter-chain counterparts [18]. Functions of VLC-PUFAs can be associated with their unique physical characteristics, as their long chains allow them to simultaneously have properties characteristic of both saturated and polyunsaturated chains [22].

The very low concentrations of VLC-PUFAs in specific classes of cellular lipids of vertebrates together with the lack of reference standards has made the characterization of these compounds challenging. Gas chromatography coupled to mass spectrometry (GC-MS) with electron ionization (EI) is the most commonly used technique for analyzing VLC-PUFAs [15]. Nevertheless, the extensive fragmentation of these compounds in the EI source leads to the partial or total loss of diagnostic molecular ions (M+•) from the spectrum [24]. In addition, the low resolution of simple quadrupole analyzers commonly used until the last decade has made difficult the identification of VLC-PUFAs. Yet, APCI has been revealed in the last years as a deserving rival to the widely

accepted EI source, as it promotes the formation of an abundant molecular ion (M+•) and/or protonated molecule ([M+H]+). This results in: i) a better sensitivity/specificity in target SRM methodologies, and ii) enhanced identification capabilities, especially when coupled to high resolution mass spectrometers (HRMS) as hybrid quadrupole time-of-flight (QTOF) [25-27]. The late elution of VLC-PUFAs supposes an additional problem because their chromatographic separation is challenging due to the column degradation after the separation of a high amount of short-chain fatty acids.

In this work, we have developed advanced methodology for the identification of VLC-PUFAs, based on GC-APCI-QTOF MS, in biological samples. Gilthead seabream was selected because of its relevance in the Mediterranean aquaculture and because it is a valuable model to investigate how the current feed formulations containing high vegetable oil levels can impact the VLC-PUFA metabolism in farmed fish [28-29]. We herein illustrate that GC-APCI-QTOF MS, together with the use of a chromatographic column suitable for temperatures up to 400 °C, allows the detection and identification of novel VLC-PUFAs, and the assessment of qualitative differences between farmed and wild fish. As a novel application, the contribution of IMS in combination with GC-APCI-QTOF MS, as well as the role of the collisional cross-section (CCS), has been evaluated for the characterization of VLC-PUFAs with chains up to 44 carbons, reported for the first time in eyes, brain and gonads of gilthead sea bream.

Experimental

Samples and Samples preparation

As mentioned above, VLC-PUFAs are endogenously produced in vivo by the action of an enzyme called Elovl4 [6,7,10,16]. Thus, we first isolated the gene encoding an Elovl4 from gilthead seabream (*Sparus aurata*) and expressed its coding region in yeast *Saccharomyces cerevisiae* to generate a set of VLC-PUFA standards. Individual cultures of transgenic yeast containing the seabream elovl4 were grown in the presence of one of the following polyunsaturated fatty acid (PUFA) substrates: C20:2n-6, C20:3n-3, C20:3n-6, C20:4n-3, C20:4n-6, C20:5n-3, C22:4n-6, C22:5n-3, C22:6n-3 and C24:5n-3. Seabream Elovl4, like all Elovl enzymes [30], can generate a series of stepwise elongation products varying in 2-carbon units, and therefore we were able to

produce polyenes up to 36 carbons [31] of the following series: 2n-6, 3n-3, 3n-6, 4n-3, 4n-6, 5n-3, and 6n-3. These compounds can be unequivocally identified using MSD EI standard conditions and thus easily located in the chromatogram, since only the transgene (gilthead seabream elovl4) and not the *S. cerevisiae* endogenous enzymes, recognize the exogenously supplemented PUFAs as metabolic substrates.

Adult specimens of gilthead seabream were dissected and eyes, brains and gonads were collected and stored at -20°C until further analysis. Crystalline lenses were removed from eyes. Total lipids were extracted using the method of Folch et al. [32]. Subsequently, an aliquot of total lipids (~200 mg) was further developed by thin layer chromatography (TLC 20x20 silica gel G60, Merck, Darmstadt, Germany) using a polar solvent system (methyl acetate: propan-2-ol: chloroform: methanol: 0.25% (w/v) aqueous KCI (25:25:25:10:9 by vol). The three fractions where VLC-PUFAs were detected in previous analyses, namely SM, PC and CE, were scrapped off the plate [33], eluted in chloroform:methanol (2:1, v/v) containing BHT (0.01%, w/v), and used to prepare fatty acid methyl esters (FAMEs) [34]. Methyl esters (ME) were stored in hexane/BHT (0.01%) under nitrogen at -20ºC (for more details see Garlito et al. [11]). Prior to the analysis by GC-APCI-QTOF MS and GC-APCI-IMS-QTOF MS samples were dried with a gentle flow of N_2 and reconstituted in 50 µL of hexane.

The experimental procedures were performed in accordance with the principles of the European Animal Directive (86/609/EEC) for the protection of experimental animals [35] and were approved by the Ethics Committees of the Spanish National Research Council (CSIC) and the Institute of Aquaculture of Torre la Sal.

GC-APCI-(Q)TOF MS

As in a previous work [11], an Agilent 7890 N gas chromatograph (Palo Alto, CA, USA) equipped with an Agilent 7683 autosampler was coupled to a quadrupole orthogonal acceleration time-of-flight mass spectrometer, Xevo G2 QTOF (Waters Corporation, Manchester, UK), equipped with APGC v2.0 as ionization source, working in positive APCI mode. A fused silica VF-5HT capillary column with a length of 15 m × 0.32 mm i.d. and a film thickness of 0.10 µm (J&W Scientific, Folson, CA, USA) was used for GC separation. The oven temperature was programmed as follows: 130 °C (1 min); 20°C/min to 340°C; (2.5 min) with a total runtime of 14 min. Pulsed

splitless (25 psi) injections of 1 μL were carried out at 280°C with a splitless time of 1 min. Helium 99.999% (Praxair, Spain) was used as carrier gas at a flow of 4 mL/min.

The interface and ionization source temperatures were set to 340°C and 150°C, respectively. N_2 was used as auxiliary gas at 200 L/h, as cone gas at 5 L/h and as make-up gas at 300 mL/min. The APCI corona discharge pin was operated at 1.7 μA and the cone voltage was set to 20 V.

The QTOF was operated at 2.5 spectra/s acquiring the mass range m/z 50–1000. The TOF MS resolution was approximately 15,000 (FWHM) at m/z 264. Acquisition was done in MSE mode in which two alternating acquisition functions with different collision energies were generated: the low-energy (LE) function, selecting a collision energy of 4 eV to avoid or minimize fragmentation, and the high-energy (HE) function, with a collision energy ramp ranging from 25 to 40 eV to obtain a greater range of fragment ions.

Heptacose (Sigma Aldrich, Madrid, Spain) was used for the daily mass calibration. Internal calibration was performed using octafluoronaphthalene (Sigma Aldrich, Madrid, Spain) as lock mass (monitoring the molecular ion, m/z 271.9872).

In order to work under proton transfer conditions, an uncapped vial containing water was placed in a designed holder into the APCI source door to enhance protonation. MS data were acquired in centroid.

GC−APCI-IMS-(Q)TOF MS

Chromatograph and autosampler described above were interfaced to a VION IMS-QTOF mass spectrometer (Waters Corporation, Manchester, UK), equipped with APGC v2.0 as ionization source, working in positive APCI mode. Chromatographic conditions were kept as described for the previous instrument.

The APCI corona discharge pin was operated at 2.0 μA and the cone voltage was set to 20 V. The interface and ionization source temperatures were set to 350°C and 150 °C, respectively. N2 was used as auxiliary gas at 300 L/h, as cone gas at 160 L/h and as make-up gas at 275 mL/min.

MS data were acquired using the VION in high-definition (HD) MSE mode, in the range 50–1000 m/z, with N2 as the drift gas, an IMS wave velocity of 250 m/s and wave height ramp of 20–50 V. Two independent scans with different collision energies were acquired during the run: a collision energy of 6 eV for LE and a ramp of 20–56 eV for HE. HDMSE implies drift time alignment between LE and HE spectra keeping only fragment ions related

to parent ions. The LE and HE functions settings was in both cases a scan time of 0.25 s. Argon (≥99.999%) was used as collision-induced dissociation (CID) gas. For MS/MS experiments, the QTOF was operated in daughter scan mode at 2.5 spectra/s acquiring the mass range from m/z 50 to the parent ion. Acquisition was done in MS/MS mode defining different windows for each compound, with a collision energy ramp from 20 to 60 eV to obtain sufficient fragment ions. All data were examined using an accurate mass screening workflow within UNIFI informatics platform from Waters Corporation.

The (de)protonated molecule of Leucine-enkephalin, at m/z 556.2771, was used for the calibration of the instrument, which must be made in electrospray ionization mode. Internal calibration was performed using two bleeding ions as lock mass (monitoring the molecular ions, m/z 355.06693 and 223.06365 corresponding to decamethylcyclopentasiloxane and hexamethylcyclotrisiloxane, respectively). Prior to the standard injection, the instrument was calibrated both for m/z measurements and CCS calculation following the manufacturer instructions. Then a 'system suitability test' (SST) containing 9 compounds was injected ten times (i.e. n=10) to check the accuracy of the instrument measurements.

Results and discussion

Detection and identification of VLC-PUFAs

From our previous experience [11] and taking into account the literature regarding PUFAs analysis by GC [8,24,25,27], three chromatographic columns (DB-5MS 60 m × 0.25 mm, 0.25 μm; BD-EN14103 30 m × 0.32 mm, 0.25 μm; VF-5HT 15m × 0.32 mm, 0.10 μm) were tested. The best results were obtained with the VF-5HT column, which was selected to continue the work. The characteristics of this column allowed to work robustly at high flow rates, up to 4 ml/min, resulting in narrower peaks (around 2 s FWHM) and increased sensitivity, compared to the other columns. Under these conditions, we were able to detect chromatographic peaks (that were expected to correspond to novel VLC-PUFAs) that were not observed using longer column with lower maximum oven temperatures [11]. Table S1 summarizes column properties and outputs.

The investigation of novel VLC-PUFAs (>C38) observed in the studied biological matrices (brain, retina and gonads) was conducted looking for a chromatographic peak for each of the corresponding [M+H]+ ions using

0.02 Da narrow window eXtracted Ion Chromatograms (nw-XICs). Fig. 1 shows VLC-PUFAs (C38:6-ME-C44:6-ME) detected in several matrices.

The MSE spectra of the different VLC-PUFAs peaks were studied for further identification of the compounds. The mass accuracy of [M+H]+ was evaluated in the low-energy (LE) function, and mass errors were below 3.2 ppm in all cases. It is remarkable that the ions observed corresponded to the protonated molecule in all cases, while M+·was not observed, a fact that sometimes occurs in GC-APCI-MS based methods [25-27]. This can be explained due to the protonable character of these compounds together with the presence of a vial with water in the APCI source, precisely to promote the formation of the protonated molecules.

Unfortunately, the fragmentation observed in HE function was found to be insufficient for identification purposes. This made unfeasible the application of ion ratio rule developed in our previous work for up to C34 VLC-PUFA [11]. Such evaluation of the ion ratios in HE spectra of analogous shorter chain (<C24) PUFAs, had allowed us to predict the positions of key double bonds, enabling the tentative classification of these compounds as n-3 or n-6.

Taking advantage of the full capabilities of the hybrid QTOF analyzer, several experiments were performed selecting $[M+H]^+$ as precursor ions, and using a collision energy ramp. The results obtained improved the possibilities for reliable identification because accurate mass fragment ions, compatible with the chemical structure, were obtained in all cases with low mass errors. Fig. 2 shows MS/MS spectrum for $[M+H]^+$ of C42:6-ME ($C_{43}H_{75}O_2$, m/z 623.576). Fragmentation routes based on intramolecular nucleophilic substitution suggest that the detected C42:6-ME is n-3 compound, which is consistent with previous findings in which all the >C24 VLC-PUFAs detected in fish were n-3 species [11]. Fig S1 shows the proposed fragmentation routes that would support this hypothesis. This behavior was also observed for the rest of VLC-PUFAs. Similarly, Fig. S2 shows MS/MS spectrum for $[M+H]^+$ of C40:6-ME ($C_{41}H_{71}O_2$, m/z 623.576)). Table 1 shows the new VLC-PUFAs found in all matrices, including molecules with chains between 38 and 44 carbons. These are present in different lipids classes of the brain, eye and gonads of gilthead sea bream.

Contribution of IMS in combination with GC-APCI-QTOF MS to the characterization of VLC-PUFAs

Ion mobility spectrometry (IMS) is a powerful technique that has been revisited in the last years [37-38]. IMS separates ions drifting through a tube filled with buffer gas in a weak electric field according to their shape-to-charge ratio. IMS provides an additional separation to the chromatographic system: ions are separated on the basis of the size-to-charge ratio in the IMS dimension and on the mass-to-charge ratio in the MS dimension. The mobility of an ion is determined by its mass and charge, and by the collisional cross section (CCS), a specific parameter valuable for compound identification that represents the rotationally averaged surface area of an ion [39].

The incorporation of IMS in hyphenated chromatography-mass spectrometry methods significantly improves peak capacity, enabling the separation of isomers, isobars and conformers, as well as reducing background noise, especially when using high-resolution mass spectrometry (HRMS). IMS combined to LC-MS has been recently applied in different fields thanks to the development of several commercial systems, with the ion mobility spectrometers based on drift time IMS (DTIMS) or travelling wave IMS (TWIMS) being the most commonly used [38,40-43]. On the contrary, the coupling of IMS with GC-MS remains practically unexplored [44]. However, GC can be efficiently coupled to IMS-QTOF making use of an APCI source (GC-APCI-IMS-QTOF), a powerful combination that has been little studied until now.

In this work, the use of CCS in a GC-APCI-IMS-QTOF instrument has been of help for VLC-PUFA identification [40,45]. This is of especial relevance when reference standards are not available for the final identification. CCS data were automatically translated by the software from drift time values prior instrument calibration to construct the translating equation. Experimental CCS (Table S2) were obtained from the injection of elongation products (see Samples and Samples preparation section). This enabled, apart from identification and retention time confirmation of VLC-PUFAs up to C38, the creation of linear regression models correlating the CCS value with the number of carbons for a given number of unsaturation's and position of the double bounds. It can be seen (Fig. S3) the good correlation between the number of carbons of the VLC-PUFA and the observed CCS, regardless of the unsaturation degree, double bond position and elongation substrate, with correlation factors greater than 0.99 in all cases. Taking into account that CCS value can differ up to a 2% in replicate

analyses of the same compound [40,46], data represented in Fig. S3 suggest that CCS models can clearly distinguish VLC-PUFA with the same number of carbons and with a difference of, at least, 2 unsaturations.

The created CCS model for 6n3 series was finally applied to the detected C40 and C42 species in fish organs in order to test whether the observed CCS values correlated well to the model. As can be appreciated in Fig. 3 observed CCS values for detected C40 and C42 fall just above the predicted curve, and below the superior error limit, confirming they belong to the VLC-PUFA family.

These models are also helpful to avoid reporting false positives, such as other isomers that might be present with the same exact mass. This would be the case of three reported isomers of C42:6n3-ME ($C_{43}H_{75}O_2$) in LipdMaps® and Pubchem databases (i.e. cholest-5-en-3β-yl (9Z-hexadecenoate), cholest-5-en-3β-yl (7Z-hexadecenoate) and (5Z, 7E)-(3S)-9,10-seco-5,7,10(19)-cholestatriene-3-yl hexadecanoate (Vitamin 3 Palmitate). Although these isomers, derived from oleic acid, could be discarded by GC retention time, their different spatial conformation would also imply notable differences in the CCS values compared to C42:6n3-ME.

Biological interpretation

The analyses carried out in organs and lipid fractions studied from both cultured and wild fish, except 42:6n-3 that was absent in the SM from the gonads and eyes, and present only in the PC from the gonads. Interestingly, the longer chain 44:6n-3 was only found in the PC fraction of the brain of wild specimens (Table 1). These results are particularly important in light of published information [18-21], since it has been suggested that the presence of adequate amounts of these compounds can impact the health and performance of farmed species. Such differences may reflect the influence of environmental conditions, especially diet, on the composition of VLC-PUFAs in brain, eye and gonads [28,29,46] and highlight the potential negative impacts of current diet formulations that lack sufficient essential VLC-PUFAs precursors (i.e. C20-22 PUFA) due to fishmeal and fish oil substitution, on neural function and reproduction. The results from the present work, open new perspectives in our understanding of VLC-PUFAs and their impact on basic biological functions. In a practical scenario like fish farming deficiency of key VLC-PUFAs can translate into altered visual acuity (critical in visual predators

including most cultured fish species), fertility issues of broodstock, and disruptions of brain functioning that can affect growth performance and eventually the economic profits.

Conclusions

In this research, we have developed advanced analytical methodology based on GC-APCI-QTOF MS for reliable identification of VLC-PUFAs. The methodology developed has allowed, for the first time, the identification of VLC-PUFAs with chains up to 44 carbons long in the SM and PC of the eyes, brain and gonads of gilthead sea bream based on their accurate mass and fragmentation pattern.

The quality of the analytical information obtained is the result of the high mass accuracy attainable with QTOF instrument, together with the soft ionization of the APCI source for GC, which provides valuable information from the intact molecule, combined with a thorough sample treatment process and optimized chromatographic separation. The application of ion mobility separation in the GC-HRMS system offers the possibility to use the CCS values to help to confirm that compounds detected did belong to the VLC-PUFA family. Additionally, the full characterization of the abovementioned substrates with very long carbon chains can provide useful information for the scientific community. The application of this method has allowed the assessment of qualitative differences between farmed and wild fish, opening new perspectives in a still incipient field of research involving the role of VLC-PUFAs.

Acknowledgements

This work has been developed within the framework of the Research Unit of Marine Ecotoxicology (IATS-CSIC/IUPA-UJI). Tania Portolés acknowledges Ramon y Cajal Program from the Ministry of Economy and Competitiveness, Spain (RYC-2017-22525) for funding her research. Carlos Sales acknowledges the financial support from University Jaume I, Spain for his predoctoral grant. Funding: Pla 2018 de Promoció de la Investigació a l'UJI. Research Project: Identificación y cuantificación de ácidos grasos poliinsaturados de cadena muy larga mediante cromatografía de gases acoplada a espectrometría de masas de alta resolución en peces. Ref: UJI-B2018-08. Programa Estatal de Investigacion, Desarrollo e Innovacion orientada a los retos de la sociedad

(Ministry of Economy and Competitiveness): Research Project “Acidos grasos poliinsaturados de cadena muy larga en peces: biosíntesis e implicaciones durante fases tempranas de desarrollo en especies cultivadas” (AGL2013-40986-R).

We are grateful with David Fabregat Safont for his help on the fragmentation routes proposal.

Appendix A. Supplementary data

Supplementary_data.docx include: Tables S1 and S2, and Figs. S1, S2 and S3

References

[1] Poulos A, Sharp P, Johnson D, White I, Fellenberg A. The occurrence of polyenoic fatty acids with greater than 22 carbon atoms in mammalian spermatozoa. Biochem J. 1986;240:891-5.

[2] Poulos A, Johnson DW, Beckman K, White IG, Easton C. Occurrence of unusual molecular species of sphingomyelin containing 28-34 carbon polyenoic fatty acids in ram spermatozoa. Biochem J. 1987;248:961-4.

[3] Aveldano MI, Sprecher H. Very long chain (C24 to C36) polyenoic fatty acids of the n-3 and n-6 series in dipolyunsaturated phosphatidylcholines from bovine retina. J. Biol. Chem. 1987;262:1180-6.

[4] Poulos A, Sharp P, Johnson D, Easton C. The occurrence of polyenoic very long chain fatty acids with greater than 32 carbon atoms in molecular species of phosphatidylcholine in normal and peroxisome-deficient (Zellwerger’s syndrome) brain. Biochem. J. 1988;253:645-50.

[5] Furland NE, Maldonado EN, Aveldaño MI. Very long chain PUFA in murine testicular triglycerides and cholesterol esters. Lipids. 2003;38:73-80.

[6] Agbaga M-P, Brush RS, Mandal MNA, Henry K, Elliot MH. Anderson R.E., Role of Stargardt-3 macular dystrophy protein (ELOVL4) in the biosynthesis of very long chain fatty acids. Proc. Natl. Acad. Sci. U. S. A. 2008;105:12843-8.

[7] Agbaga M-P, Mandal MNA, Anderson RE. Retinal very long-chain PUFAs: new insights from studies on ELOVL4 protein. J. Lipid Res. 2010;51:1624–42.

[8] Liu A, Chang J, Lin Y, Shen Z, Bernstein PS. Long chain and very long-chain polyunsaturated fatty acids in ocular aging and age-related macular degeneration. J. Lipid Res. 2010;51:3217-29.

[9] Liu A, Terry R, Lin Y, Nelson K, Bernstein PS. Comprehensive and sensitive quantification of long-chain and very long-chain polyunsaturated fatty acids in small samples of human and mouse retina. J. Chromatogr. A. 2013;1307:191-200.

[10] Deák F, Anderson RE, Fessler JL, Sherry DM. Novel Cellular Functions of Very Long Chain-Fatty Acids: Insight From ELOVL4 Mutations. Front. Cell. Neurosci. 2019;13:428.

[11] Garlito B, Portolés T, Niessen WMA, Navarro JC, Hontoria F, Monroig O, Varó I, Serrano R. Identification of very long-chain (>C24) fatty acid methyl esters using gas chromatography coupled to quadrupole/time of flight mass spectrometry with atmospheric pressure chemical ionization source. Anal. Chim. Acta. 2019;1051:103-9.

[12] Poulos A. Very long chain fatty acids in higher animals—A review. Lipids. 1995;30:1–14.

[13] Harkewicz R, Du H, Tong Z, Alkuraya H, Bedell M, Sun W, Wang X, Hsu YH, Esteve-Rudd J, Hughes G, Su Z, Zhang M, Lopes VS, Molday RS, Williams DS, Dennis E, Zhang K. Essential role of ELOVL4 protein in very long chain fatty acid synthesis and retinal function. J. Biol. Chem. 2012;287:11469–80.

[14] Barabas P, Liu A, Xing W, Chen C-K, Tong Z, Watt CB, Jones BW, Bernstein PS, Križaj D. Role of ELOVL4 and very long-chain polyunsaturated fatty acids in mouse models of Stargardt type 3 retinal degeneration. Proc. Natl. Acad. Sci. U. S. A. 2013;110:5181–6.

[15] Tejada-Casado C, Hernández-Mesa M, Monteau F, Lara FJ, del Olmo-Iruela M, Garcia-Campaña AM, Le Bizec B, Dervilly-Pinel G. Collision cross section (CCS) as a complementary parameter to characterize human and veterinary drugs. Anal. Chim. Acta. 2018;1043:52–63.

[16] Castro LFC, Tocher DR, Monroig O. Long-chain polyunsaturated fatty acid biosynthesis in chordates: Insights into the evolution of Fads and Elovl gene repertoire. Prog Lipid Res. 2016;62:25-40.

[17] Paglia G, Angel P, Williams JP, Richardson K, Olivos HJ, Thompson JW, Menikarachi L, Lai S, Walsh C, Moseley A, Plumb RS, Grant DF, Palsson BO, Langridge J, Geromanos S, Astarita G. Ion-mobility-derived collision cross section as an additional measure for lipid fingerprinting and identification. Anal. Chem. 2017;87:1137–44.

[18] McMahon A, Jackson SN, Woods AS, Kedzierski WA. A Stargardt disease-3 mutation in the mouse Elovl4 gen causes retinal deficiency of C-32-C36 acyl phospathidylcholines. FEBS Lett. 2007;581:5459-63.

[19] Monroig O, Rotllant J, Cerdá-Reverter JM, Dick JR, Figueras A, Tocher DR. Expression and role of Elovl4 elongases in biosynthesis of very long-chain fatty acids during zebrafish *Danio rerio* early embryonic development. Biochim. Biophys. Acta - Mol. Cell Biol. Lipids. 2010;1801:1145–54.

[20] Monroig Ó, Webb K, Ibarra-Castro L, Holt GJ, Tocher DR. Biosynthesis of long-chain polyunsaturated fatty acids in marine fish: Characterization of an Elovl4-like elongase from cobia *Rachycentron canadum* and activation of the pathway during early life stages. Aquaculture. 2011;312:145-53.

[21] Carmona-Antonanzas G, Monroig Ó, Dick JR, Davie A, Tocher DR. Biosynthesis of very long-chain fatty acids (>24) in Atlantic salmon: Cloning, functional characterization, and tissue distribution of an Elovl4 elongase. Comp. Biochem. Physiol. B. 2011;159:122-9.

[22] Monroig Ó, Wan SQ, Zhang L, You CH, Tocher DR, Li Y. Elongation of long-chain fatty acids in rabbitfish *Signatus canaliculatus*: Cloning, functional characterization and tissue distribution of Elovl5 and Elovl4 like elongases. Aquaculture. 2012;350:36-70.

[23] Pettersen J. Determination of trans double bonds in polyunsaturated fatty acid methyl esters from their electron impact mass spectra. Research Paper. 2003;105:156–64.

[24] Kloos DP, Gay E, Lingeman H, Bracher F, Müller C, Mayboroda O, Deelder AM, Niessen WM, Giera M. Comprehensive gas chromatography-electron ionisation mass spectrometric analysis of fatty acids and sterols using sequential one-pot silylation: quantification and isotopologue analysis. Rapid Commun. Mass Spectrom. 2014;28:1507–14.

[25] Portolés T, Ibáñez M, Garlito B, Nácher-Mestre J, Karalazos V, Silva J, Alm M, Serrano R, Pérez-Sánchez J, Hernández F, Berntssen MHG. Comprehensive strategy for pesticide residue analysis through the production cycle of gilthead sea bream and Atlantic salmon. Chemosphere. 2017;179:242-53.

[26] Hernández F, Ibáñez M, Portolés T, Cervera MI, Sancho JV, Benet FL. Advancing towards universal screening for organic pollutants in waters. J. Hazard. Mat. 2015,;282:86 – 95.

[27] Portolés T, Mol JGJ, Sancho JV, Hernández F. Validation of a qualitative screening method for pesticides in fruits and vegetables by gas chromatography quadrupole time-of-flight mass spectrometry with atmospheric pressure chemical ionization. Anal. Chim. Acta. 2014;838:76 - 85.

[28] Torres M, Navarro JC, Varó I, Agulleiro MJ, Morais S, Monroig O, Hontoria F. Expression of genes related to long-chain (C18-22) and very long-chain (>C24) fatty acid biosynthesis in gilthead seabream (*Sparus aurata*) and Senegalese sole (*Solea senegalensis*) larvae: Investigating early ontogeny and nutritional regulation. Aquaculture. 2020;520:734949.

[29] Torres M, Navarro JC, Varó I, Monroig O, Hontoria F. Nutritional regulation of genes responsible for long-chain (C20-24) and very long-chain (>C24) polyunsaturated fatty acid biosynthesis in post-larvae of gilthead seabream (*Sparus aurata*) and Senegalese sole (*Solea senegalensis*). Aquaculture. 2020;525:735314.

[30] Castro LFC, Tocher DR, Monroig O. Long-chain polyunsaturated fatty acid biosynthesis in chordates: Insights into the evolution of Fads and Elovl gene repertoire. Prog Lipid Res. 2016;62:25-40.

[31] Morais S, Torres M, Hontoria F, Monroig Ó, Varó I, Agulleiro MJ, Navarro JC. Molecular and functional characterization of elovl4 genes in *Sparus aurata* and *Solea senegalensis* pointing to a critical role in very long-chain (>C24) fatty acids synthesis during early neural development of fish. Int J Molecular Sci. submitted

[32] Folch J, Lees M, Stanley GHSJ. A simple method for the isolation and purification of total lipids from animal tissues. Biol. Chem. 1957;226:497–509.

[33] Christie WWJ. Esterification of fatty acids in adipose-tissue. Sci. Food Agr. 1982;33:809.

[34] Navarro JC, Batty RS, Bell MV, Sargent JR. Rffects of two Artemia diets with different contents of polyunsaturated fatty acids on the lipid composition of larvae of Atlantic herring (Clupea harengus). J. Fish Biol. 1993;43:503-15.

[35] Comission E. COUNCIL DIRECTIVE of 24 November 1986 on the approximation of laws, regulations and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific purposes. 1986;86/609/ECC(L358/1).

[36] Sobrado L, Freije-Carrelo L, Moldovan L, Ruiz-Encinar J, Garcia JI. Comparison of gas chromatography-combustion-mass spectrometry and gas chromatography-flame ionization detector for the determination of fatty acid methyl esters in biodiesel without specific standards. J. Chromatogr. A. 2016;1457:134-43.

[37] Armenta S, Alcala M, Blanco M. A review of recent, unconventional applications of ion mobility spectrometry (IMS). Anal. Chim. Acta. 2011;703:114-23.

[38] Cumeras R, Figueras E, Davis CE, Baumbach JI, Gràcia I. Review on Ion Mobility Spectrometry. Part 1: current instrumentation. Analyst. 2015;140:1376-85.

[39] Kanu AB, Dwivedi P, Tam M, Matz L, Hill HHJ. Ion mobility−mass spectrometry. J. Mass Spectrom. 2008;43:1-22.

[40] Bijlsma L, Bade R, Celma A, Mullin L, Cleland G, Stead S, Hernández F, Sancho JV. Prediction of Collision Cross-Section Values for Small Molecules: Application to Pesticide Residue Analysis. Anal. Chem. 2017;89:6583–89.

[41] Paglia G, Astarita G. Metabolomics and lipidomics using traveling-wave ion mobility mass spectrometry. Nature Protocols. 2017;12:797-813.

[42] Paglia G, Angel P, Williams JP, Richardson K, Olivos HJ, Thompson JW, Menikarachi L, Lai S, Walsh C, Moseley A, Plumb RS, Grant DF, Palsson BO, Langridge J, Geromanos S, Astarita G. Ion-mobility-derived collision cross section as an additional measure for lipid fingerprinting and identification. Anal. Chem. 2017;87:1137–44.

[43] Levy A. J., Oranzi N.R., Ahmadireskety A., Kemperman R.H.J., Wei M. S., Yost R.A., Tian L.L.; Zeng Y.Y.; Zheng X.Q.; Chiu Y.H.; Liu T., Recent progress in metabolomics using ion mobility-mass spectrometry. TrAC - Trends in Analytical Chemistry. 2019;116:274-81,

[44] Lipok C, Hippler J, Schmitz OJ. A four dimensional separation method based on continuous heart-cutting gas chromatography with ion mobility and high resolution mass spectrometry. J. Chromatogr. A. 2018;1536:50-57.

[45] Tian L, Zeng Y, Zheng X, Chiu Y, Liu T. Detection of peanut oil adulteration mixed with rapeseed oil using gas chromatography and gas chromatography-ion mobility spectrometry. Food Anal. Methods. 2019;12:2282–92.

[46] Li S, Monroig Ó, Wang T, Yuan Y, Navarro JC, Hontoria F, Liao K, Tocher DR, Mai K, Xu W, Ai Q. Functional characterization and differential nutritional regulation of putative Elovl5 and Elovl4 elongases in large yellow croaker (*Larimichthys crocea*). Sci. Rep. 2017;7.

Figure Captions

Fig. 1 GC-APCI-QTOF MS nw-XICs for new VLC-PUFAs detected in each of the studied matrices: (A) brain, (B) retina and (C) gonads. TIC: Total Ion Chromatogram.

Fig. 2 GC-APCI-QTOF MS/MS product ion scan (CE ramp 20-60 eV) spectra for the ion m/z 623, corresponding to C42:6n-3 identified in PC of the brain of wild gilthead sea bream.

Fig. 3 Correlation between number of carbons in the chain for 6n3 VLC-PUFAs with its calculated upper and lower error limits, and experimental values for detected C40:6 and C42:6.

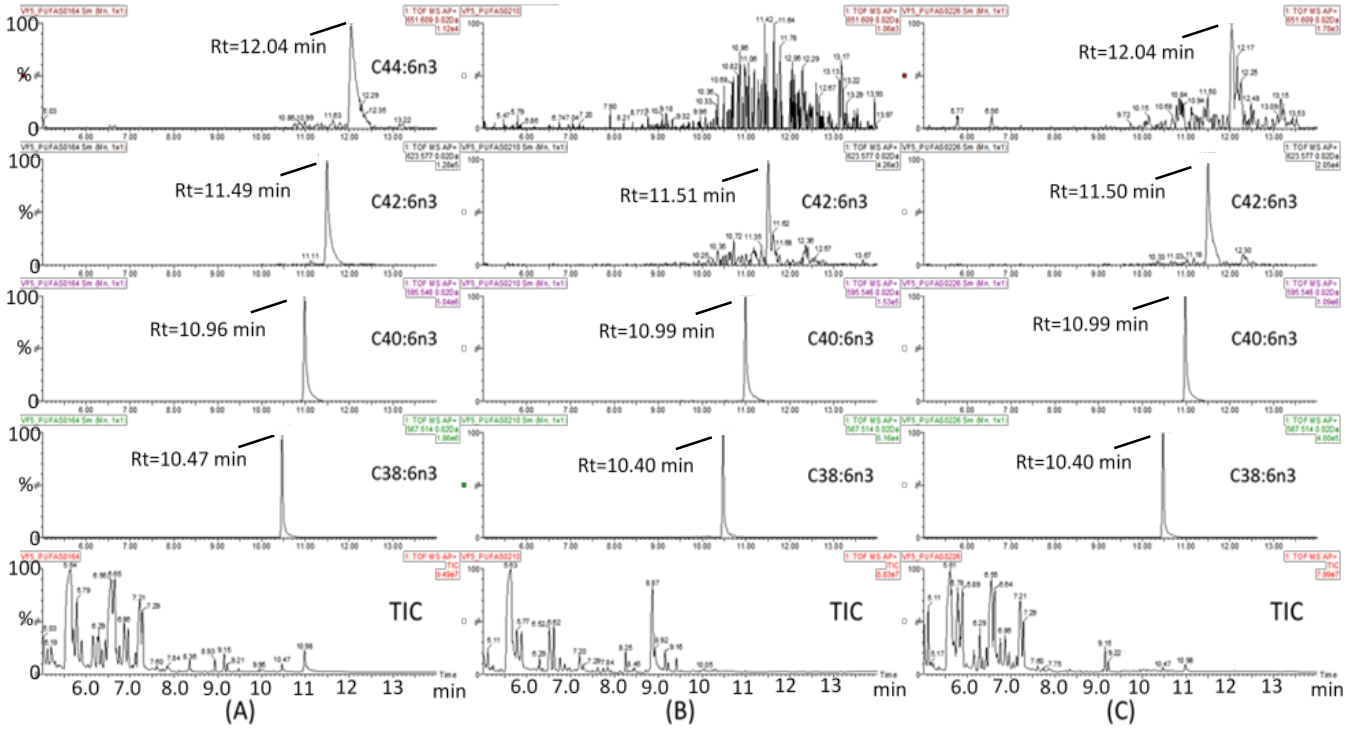
Rt=12.04 min
C44:6n3
Rt=11.49 min
C42:6n3
Rt=10.96 min
C40:6n3
Rt=10.47 min
C38:6n3
TIC
Rt=11.51 min
C42:6n3
Rt=10.99 min
C40:6n3
Rt=10.40 min
C38:6n3
TIC
Rt=12.04 min
Rt=11.50 min
C42:6n3
Rt=10.99 min
C40:6n3
Rt=10.40 min
C38:6n3
TIC
100
%
0
6.0 7.0 8.0 9.0 10 11 12 13 min
(A)
(B)
(C)

Figure 1

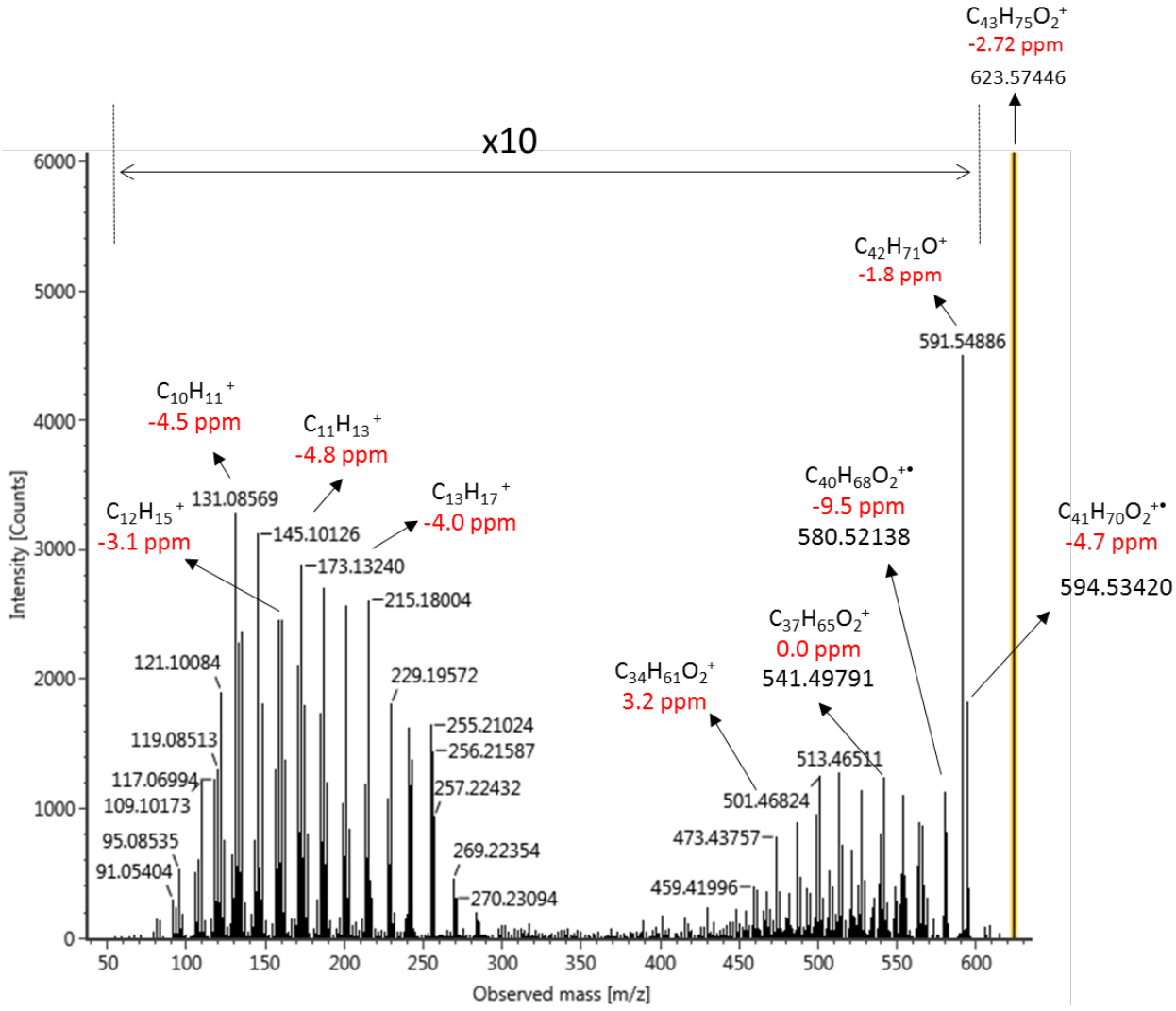
C43H75O2+
-2.72 ppm
623.57446
x10
C42H71O+
-1.8 ppm
591.54886
C10H11+
-4.5 ppm
C11H13+
-4.8 ppm
C12H15+
-3.1 ppm
131.08569
145.10126
173.13240
C13H17+
-4.0 ppm
215.18004
C40H68O2+•
-9.5 ppm
580.52138
C41H70O2+•
-4.7 ppm
594.53420
C37H65O2+
0.0 ppm
541.49791
C34H61O2+
3.2 ppm
121.10084
229.19572
119.08513
255.21024
256.21587
117.06994
257.22432
109.10173
95.08535
269.22354
91.05404
270.23094
501.46824
513.46511
473.43757
459.41996
Intensity [Counts]
Observed mass [m/z]
0
1000
2000
3000
4000
5000
6000
50
100
150
200
250
300
350
400
450
500
550
600

Figure 2

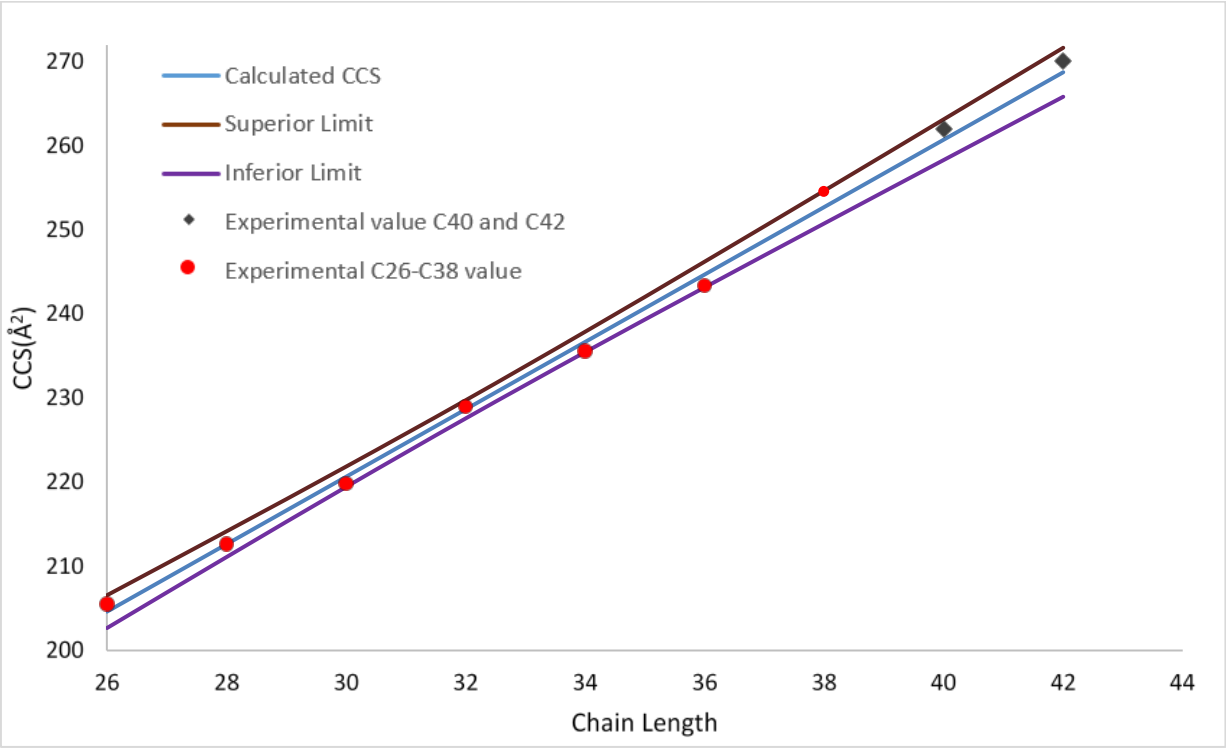
Calculated CCS
Superior Limit
Inferior Limit
Experimental value C40 and C42
Experimental C26-C38 value
CCS(Å²)
270
260
250
240
230
220
210
200
26
28
30
32
34
36
38
40
42
44
Chain Length

Figure 3

Table 1

List of new VLC-PUFAs identified in the different classes of lipids in the brain, eye and gonads of wild and cultured gilthead sea bream

	Brain		Eye			Gonads	
Compound	SM	PC	SM	PC	CE	SM	PC
C38:6n-3	**	**	**	**	*	**	**
C40:6n-3	**	**	**	**		**	**
C42:6n-3	**	**		**			*
C44:6a		*					

*SM: Sphingomyelin; PC: Phosphatidylcholine; CE: Cerebrosides; ** detected in wild and cultured fish; * detected only in wild fish. a: C44:6 was characterized by GC-APCI-QTOF, based on only on the accurate mass of the protonated molecule. No fragmentation information was obtained due to the low concentration of the analyte. No IMS information was obtained due to sample limitations.*